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Tramadol regulates proliferation, migration and invasion via PTEN/PI3K/AKT signaling in lung adenocarcinoma cells

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Abstract. – OBJECTIVE: Tramadol is used mainly for the treatment of moderate to severe chronic cancer pain. However, the effect of tramadol on lung cancer remains unclear. Therefore, it is important to explore the mechanism accounting for the function of tramadol on lung cancer.

MATERIALS AND METHODS: We investigated the effects of tramadol on the proliferation, migration and invasion in human lung adenocarcinoma cells *in vitro* by CCK-8 assay, wound healing assay and Transwell assay, respectively. We also explored the potential mechanism of tramadol on lung cancer cells by Western blotting.

RESULTS: A549 and PC-9 cells were incubated with 2 μM tramadol for different time (0, 7, 14 and 28 d). The *in vitro* experiments showed that tramadol treatment significantly inhibited cell proliferation, migration and invasion in a time-dependent manner. Moreover, administration of tramadol suppressed tumor growth *in vivo*. The data also revealed that tramadol could up-regulate the protein expression level of PTEN and consistently inhibit the phosphorylation level of PI3K and Akt, whereas the total level of PI3K and Akt remain unchanged.

CONCLUSIONS: These findings indicated that tramadol inhibited proliferation, migration and invasion of human lung adenocarcinoma cells through elevation of PTEN and inactivation of PI3K/Akt signaling.

Key Words:
Tramadol, Lung adenocarcinoma cells, PTEN, PI3K/Akt.

Introduction

Lung cancer is a major cause of malignancy-related deaths in both men and women worldwide, and the 5-years survival rate is only 8-14%[1]. Non-small cell lung cancers (NSCLC), including adenocarcinomas, accounts for 80% of all cases of lung cancers[2]. Surgical resection is a commonly used therapy for patients with non-small cell lung carcinoma. Unfortunately, recurrence and metastasis after lung cancer surgery are widespread, which are major causes of treatment failure in patients with lung adenocarcinoma[3,4].

Tramadol, a synthetic analog of codeine, is used widely as a well-tolerated opioid analgesic for the treatment of postoperative pain, chronic non-cancer pain and cancer-related pain[5-7]. Two main mechanisms contribute to the antinociceptive effects of tramadol: the activation of μ opioid receptors[8,9] and the blocking of serotonin (5-HT) and noradrenaline reuptake[10]. Recently, increasing studies show that anesthetics, such as morphine[11], propofol[12] and sevoflurane[13,14] have an impact on the malignancy of tumor cells *in vitro*, which should be used for cancer surgery in order to decrease the risk of recurrence and metastasis after cancer surgery[15,16]. More important, tramadol has been found to inhibit the tumorgenesis and carcinogenesis of breast cancer cells through α2-adrenoceptor signaling[17], suggested the mechanism underlying the tramadol induced anti-tumor effect. However, there were few reports about tramadol on lung cancer. The function and mechanism of tramadol on lung cancer cells need further investigation.

Phosphatase and tensin homolog (PTEN) is well known as a bona fide tumor suppressor which is frequently inactivated in human malignant tumors such as prostate cancer, endometrial, pulmonary, glioblastoma, colorectal, gastric, melanoma and breast cancer[18-21]. Previous researches[22,23] demonstrated that by negative regulating phosphoinositide 3-kinase/protein kinase B (PI3K/Akt), a crucial downstream signaling pathway of PTEN, PTEN plays a critical role in tu-

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mor growth, invasiveness, differentiation, angiogenesis and chemotherapy resistance. Moreover, Pérez-Ramírez et al[24] revealed that PTEN/PI3K/Akt pathway was associated with the drug resistance and lower survival in NSCLC patients. Therefore, it is essential for us to elucidate whether tramadol has an impact on PTEN/PI3K/Akt pathway in lung cancer.

In the present study, we demonstrated that tramadol significantly suppressed the proliferation, migration and invasion of A549 and PC-9 cells *in vitro* and decreased the growth of established tumors *in vivo*. Furthermore, we also confirmed that tramadol can inactivate the PI3K/Akt signaling pathway through upregulating PTEN expression. These data indicated an important role for tramadol in regulation the malignancy of lung cancer, suggested that tramadol could be a novel therapeutic target for lung cancer.

Materials and Methods

Major Reagents

Human lung adenocarcinoma cell lines A549 and PC-9 were purchased from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Fetal bovine serum (FBS), Roswell Park Memorial Institute 1640 (RPMI-1640) medium, penicillin and streptomycin were obtained from Invitrogen (Carlsbad, CA, USA). Tramadol, protease inhibitor cocktail and MTT were purchased from Sigma Chemicals (St. Louis, MO, USA). Monoclonal primary antibodies PTEN, Akt, p-Akt-ser307, PI3K, p-PI3K, GAPDH and Horseradish peroxidase (HRP)-conjugated secondary antibodies were all purchased from Cell Signaling Technology (Danvers, MA, USA). All the other reagents were procured from Beyotime Biotechnology (Jiangsu, China).

Cell Culture

Human lung adenocarcinoma cell lines A549 and PC-9 were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were passaged habitually at 3-4 day intervals at 37°C in a humid atmosphere of 5% CO_2.

Tramadol Treatment

According to Lintz et al's study[25], the concentration of tramadol reached approximately 2 μM in human serum after an intravenous injection of 100 mg, which could be used for a clinical dosage. Consequently, in the present study, a malignancy of A549 and PC-9 cells was detected after treatment of 2 μM tramadol for different time (0, 7, 14, or 28 d).

Proliferation Assays

Cell proliferation was measured with the MTT assay in according with the manufacturer's instructions. A549 and PC-9 cells were seeded at 2×10^3 cells/well in a 96-well microtiter plates and cultured for 48 hours. Thereafter, 20 μl MTT solutions (5 mg/ml) were added to each well. The mixture was further incubated at 37°C for 4 h. After discard the supernatant, the pellet was resuspended and 150 μl dimethyl sulfoxide (DMSO) was added to each well to dissolve the precipitates, followed by agitation at room temperature for 15 min. The absorbance of each well at 570 nm was determined using a microplate ELISA reader (Molecular Devices, Silicon Valley, CA, USA).

Wound Healing Assay

The migration ability was detected by a wound healing assay. A549 and PC-9 cells were plated into 6-well plates (1×10^6 cells per well). When the cells were grown to the confluent monolayer, a scratch wound was inflicted using a sterile pipette tip across the center of the well. After removal of the floating debris, wound closure of A549 and PC-9 cells were captured respectively at two preselected time points (0 and 48 hours) using a light microscope (Olympus, Lake Success, NY, USA). Five randomly selected fields were marked of each wound, and the gap distance of migrating cells was measured (the gap width at 0 hour was set as 0%).

Transwell Invasion Assay

The effect of tramadol on the invasion of A549 and PC-9 cells was performed using transwell chambers (6.5 mm diameter and 8 μm pore size; Millipore, Billerica, MA, USA). After treated with 2 μM tramadol for various time, cells were plated onto the Matrigel-coated upper part of the transwell chamber, fetal bovine serum (FBS) medium (20%) was added to the lower wells as a chemoattractant. 48 hours later, non-invading cells were removed, the invaded cells were fixed with 4% paraformaldehyde for 30 min and stained with 1% crystal violet for 30 min. The number of stained cells on the undersurface of the polycarbonate membranes was then counted

visually in five random image fields at 200 × magnifications using a microscope (Olympus, Lake Success, NY, USA).

Western Blot Analysis

After treatment of tramadol, the whole-cell protein was prepared from A549 and PC-9 cells in RIPA Lysis Buffer supplemented with protease inhibitor cocktail. Protein concentrations were quantified using a BCA protein assay kit. 20 μg of protein samples were isolated through a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, and then electrophoretically transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membranes were then blocked with 5% skimmed milk for 1 hour at room temperature and subsequently incubated overnight at 4°C with the specific primary antibody against human PTEN (1/2000 dilution), Akt (1/2000 dilution), p-Akt-ser307 (1/1000 dilution), PI3K (1/2000 dilution), p-PI3K (1/1000 dilution), and GAPDH (1/5000 dilution). Thereafter, membranes were washed five times with PBST followed by incubation with a secondary antibody (1/5000 dilution) at room temperature for 1 h. After that, the protein bands were detected using a digital imaging system (DuPont, Boston, MA, USA) and quantified by densitometry using Image J.

In vivo Tumor Growth Assays

All male BALB/c-nu mice were purchased from College of Veterinary Medicine Yangzhou University (Yangzhou, China). Mice were maintained in specific pathogen-free (SPF) conditions for each experiment, 12 mice were randomly sorted into 2 groups: PBS-treated and tramadol-treated. Xenografts were performed by subcutaneously injecting 1×10^7 A549 cells in 200 μL volume into the right flank of mice. For the following days, mice were administered with PBS or tramadol every day for 28 days. Tumor length (a) and breadth (b) were measured in two days. Tumor volumes were quantified according to the formula: volume = length × width^2/2[26]. The mice were euthanized on day 28 and solid tumors weight was recorded. All experiments were performed according to the NIH Guidelines.

Statistical Analysis

Data were analyzed using SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). Data were expressed as mean ± standard deviation (SD). Differences in groups were compared using repeated measure analysis of variance (ANOVA). Differences among groups were analyzed using one-way ANOVA, followed by Duncan's test for post hoc comparisons. $p < 0.05$ was considered statistically significance.

Results

Tramadol Inhibits the Proliferation of Lung Adenocarcinoma Cells

To characterize the role of tramadol in proliferation, A549 and PC-9 cells were treated with 2 μM tramadol for different time (0, 7, 14 and 28 d). The inhibition of cell proliferation was detected by MTT colorimetric assay. As shown in Figure 1A and B, treatment with tramadol reduced the proliferation of A549 and PC-9 cells in a

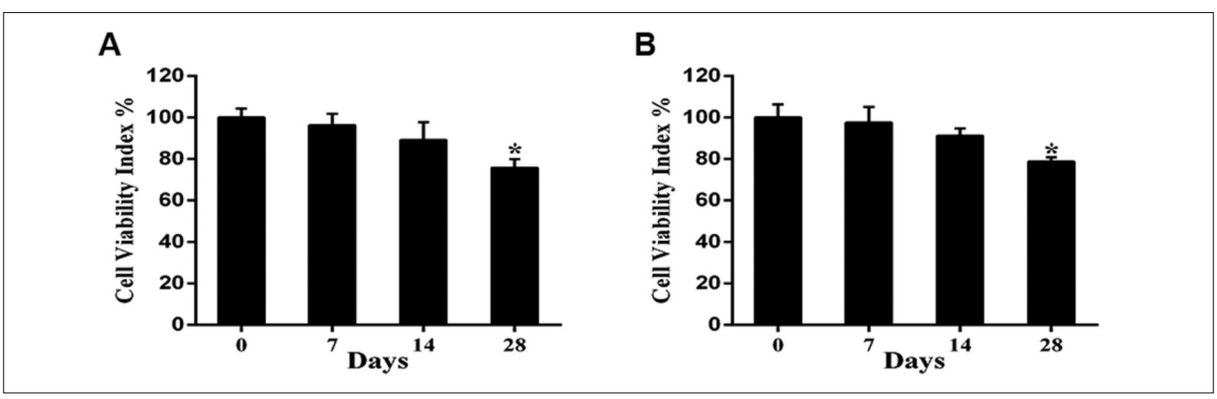

Figure 1. Tramadol inhibits proliferation of lung cancer cells *in vitro*. The proliferation rates of A549 ***(A)*** and PC-9 ***(B)*** cells were measured using a MTT assay at various time points (0, 7, 14 and 28 days) after treatment of tramadol. The absorbance at 490 nm was measured. Data are shown as mean ± SEM (n = 3), $*p < 0.05$.

time-dependent manner and peaked at 28 d. These results suggested tramadol inhibited proliferation rate of lung adenocarcinoma cells.

Administration of Tramadol Suppresses Tumor Growth In vivo

To examine anti-tumor effects caused by tramadol treatment *in vivo*, A549 cells were subcutaneously inoculated into the right flank of the mice. When tumor volume reached 100-150 mm^3, mice were treated with PBS or tramadol (20 mg/kg)[17] every day for 28 days. During the treatment periods, the tumor size was measured every 2 days for 28 days. Results showed that treated with tramadol significantly repressed tumor growth and tumor mass in contrast to the control group (Figure 2A and B), which revealed tramadol had proficient tumor inhibitory activities *in vivo*.

Tramadol Reduces the Migration of Lung Adenocarcinoma Cells

We investigated the function of tramadol on migration using wound healing assay. As shown in Figure 3A and B, in contrast to the control group, tramadol treatment continued to decrease the scratch wounds closure and inhibited the migration rate of A549 and PC-9 cells in a time-dependent manner. At 28 day, (59.91% ± 4.47%) distance of the wound was closed by cells from tramadol treatment group in A549 cells, which is markedly less than that from the control group (82.33% ± 6.81%). Similarly, in PC-9 cells, the migration rate was (64.91% ± 3.79%) post-treatment of tramadol *vs*. (86.95% ± 4.87%) of the group control. These results indicated that tramadol could repress the migration activity of lung cancer cells.

Tramadol Decreases the Invasion of Lung Adenocarcinoma Cells

To explore the effect of tramadol on the invasiveness of A549 and PC-9 cells, we evaluated the number of invasive cells using transwell assays. As demonstrated in Figure 4A and B, the number of invading cells remained decreased in the tramadol-treated group compared to the control group, indicating that tramadol suppressed the invasive capacity of the two types of cells. More important, the suppression effect of tramadol was in a time-dependent manner. These findings demonstrated an anti-invasion function of tramadol.

Tramadol Inhibits Tumor Malignancy Through PTEN/PI3K/Akt Pathway

Accumulative studies indicated that PTEN/PI3K/Akt pathway involved in tumorgenesis and carcinogenesis[22,23]. To determine whether tramadol-mediated anti-tumorigenic functions on A549 and PC-9 cells are through the inhibition of PTEN/PI3K/Akt signaling, we evaluated the effect of tramadol on the expression of PTEN, PI3K and Akt by Western blotting. Considering tramadol significantly inhibited malignancy of lung cancer cells at day 28 in our study, cells

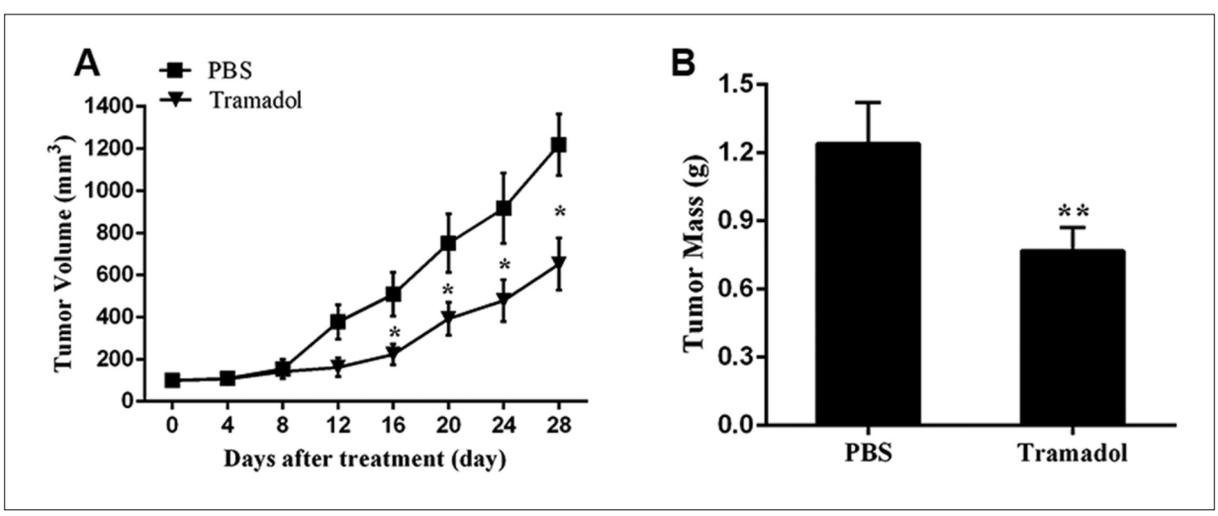

Figure 2. Tramadol suppresses migration of lung cancer cells *in vitro*. ***A,*** and ***B,*** Cell migration was evaluated by wound healing assay in A549 and PC-9 cells. Representative images were obtained at time point 0 and 48 h. Migration rate was quantified by measuring gap distance. Data are shown as mean ± SEM (n = 3), $*p < 0.05$.

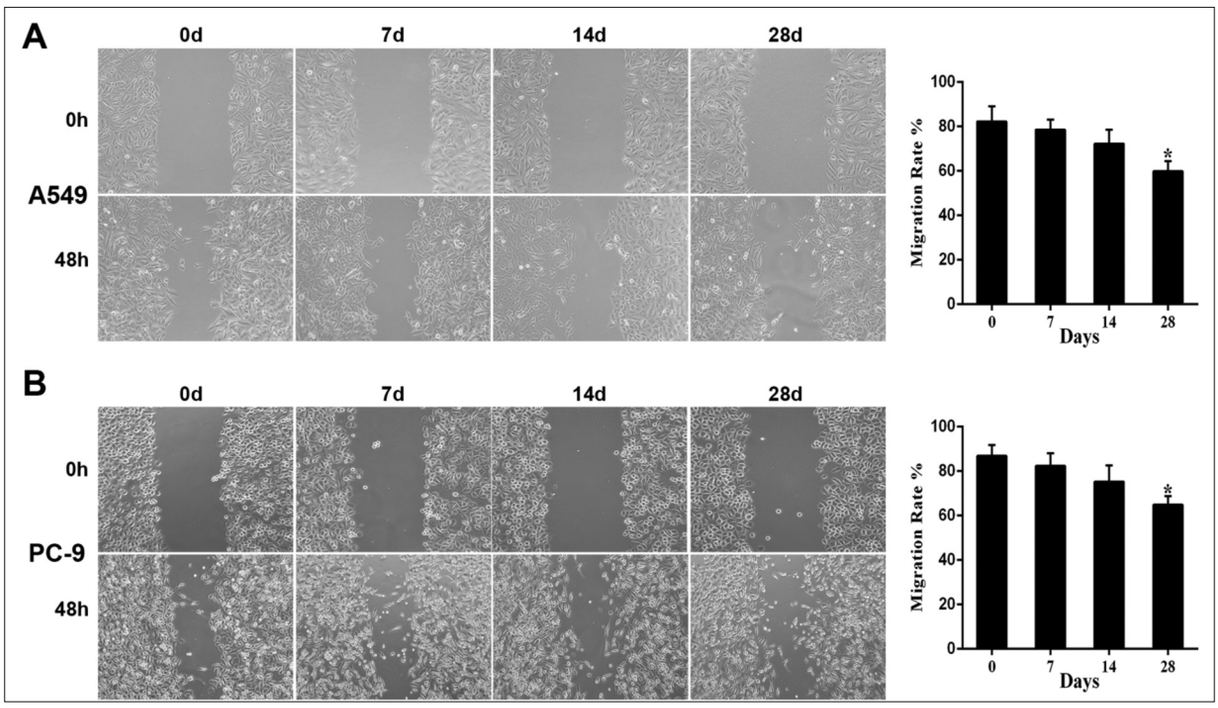

Figure 3. Tramadol decreases invasion of lung cancer cells *in vitro*. ***A,*** A549 and PC-9 cells were treated with tramadol for different time and cell invasion ability was evaluated with matrigel transwell assay. ***B,*** The bars represent the number of invasive cells in four groups. Data are shown as mean ± SEM (n=3), $*p < 0.05$.

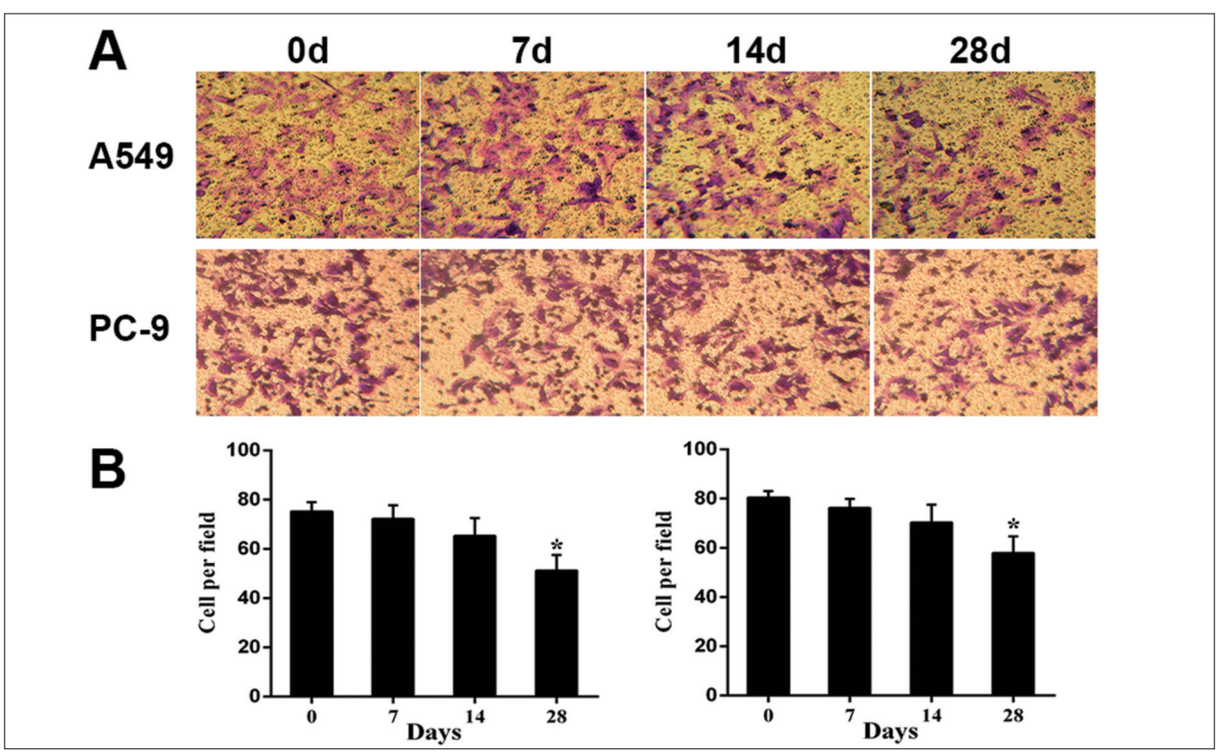

Figure 4. Tramadol inhibits the growth of established tumors in vivo. ***A,*** The volumes of tumors were recorded every 4 days after injection of pbs or tramadol. ***B,*** Mice were sacrificed and the tumors were weighted 28d after treatment of pbs or tramadol. Data are shown as mean ± SEM (n = 3), $*p < 0.05$, $**p < 0.01$.

were divided into two groups: day 0 group (group 0) and day 28 group (group 28). We first examined the protein levels of PTEN in A549 and PC-9 cells, as shown in Figure 5A and B, treatment with tramadol could increase the levels of the PTEN in two cell lines. Subsequently, we would like to further investigate the effects of the tramadol on the key members of the PTEN pathway. Interestingly, we found that the phosphor-PI3K and phosphor-AKT protein levels were also down-regulated in the same treatment of tramadol. However, the total levels of PI3K and Akt remain unchanged (Figure 6A and B). These results suggested that tramadol exerts an anti-tumor effect through affect PTEN/PI3K/Akt pathway.

Discussion

Lung cancer is still the leading cause of cancer-related deaths worldwide, with the highest mortality and morbidity rates[27]. Despite significant advances and improves made in chemotherapy and radiotherapy, the incidence in both men and women is increasing every year[28]. The major

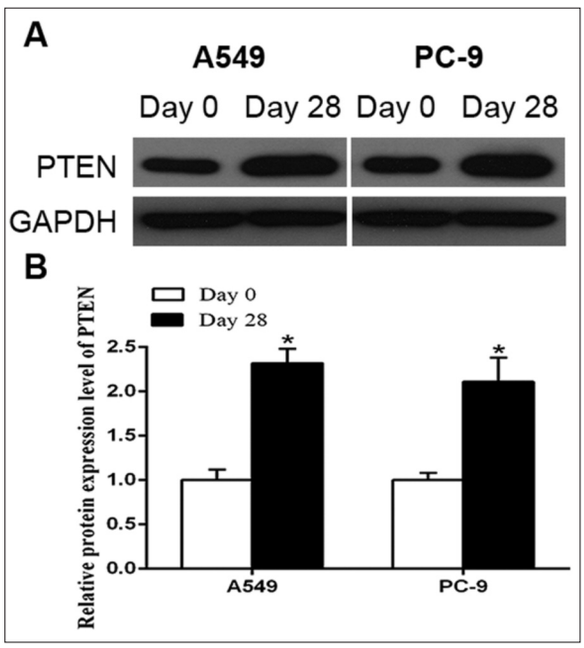

Figure 5. Tramadol increases the protein expression level of PTEN. ***A,*** Expression of PTEN was evaluated by Western blot for A549 and PC-9 cells after treated with tramadol for 28d. ***B,*** The bars represent PTEN expressions of each group. Data are shown as mean ± SEM (n=3), $*p < 0.05$.

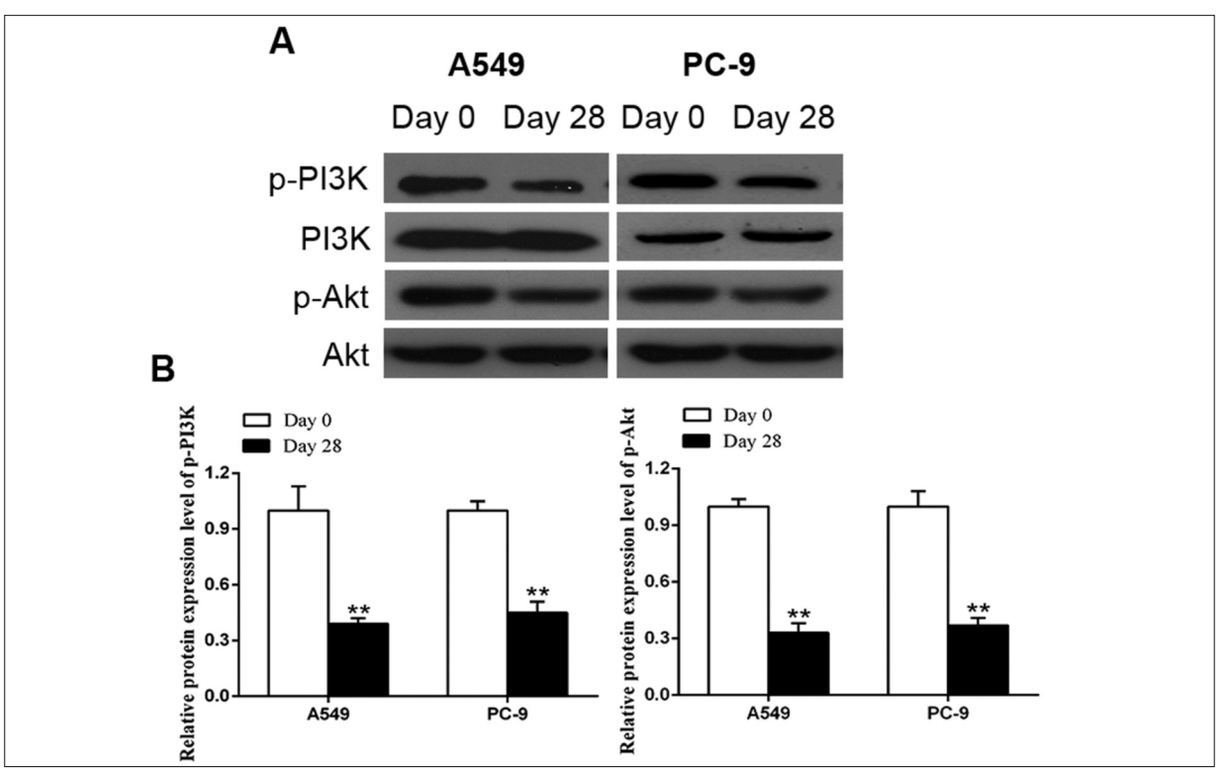

Figure 6. Tramadol inactivates the PI3K/Akt signaling pathway. ***A,*** Expression of PI3K, Akt, p-PI3K and p-Akt were evaluated by Western blot for A549 and PC-9 cells after treated with tramadol for 28d. ***A,*** The bars represent PI3K, Akt, p-PI3K and p-Akt expressions of each group. Data are shown as mean ± SEM (n = 3), $**p < 0.01$.

obstacle to the development of effective treatment is the rapid proliferation and metastasis of lung cancer. It is of great importance for us to explore new and effective therapeutic approaches.

Tramadol has been indicated to have local analgesic effect in chronic pain and cancer pain[5-7] by activates μ opioid or inhibits the reuptake of monoaminergic neurotransmitters[8,9] in the previous study. Moreover, cancer patients were treated with tramadol oral or intramuscular for periods of 14 days up to 14 months[29]. We are confused whether long time use of tramadol has an inhibitory effect on lung cancer. As we expected, in the present work, we revealed that long-term use of tramadol suppressed the proliferation, migration and invasion in a time-dependent manner *in vitro* and *in vivo*, which was in accordance with the previous studies showing that tramadol decreased the malignancy of breast cancer cells[17]. However, tramadol had limited effect on cell apoptosis in A549 and PC-9 cells, with an apoptosis rate close to 5% (data not shown). Therefore, we hypothesized that tramadol was not involved in apoptosis of lung cancer cells.

A growing number of investigations[30,31] showed that through dephosphorylating phosphatidylinositol-3,4,5-trisphosphate (PIP3) and subsequently regulate the PI3K/Akt signaling pathway, PTEN has a critical effect on the regulation of cell differentiation, cell cycle detention, migration, apoptosis and the sensitivity to cancer cells via interfering various signaling pathways. Akt is a critical downstream target of PTEN, which plays an unappreciated role in an outermost complex network of cell growth modulation that affects protein biosynthesis, cell cycle arrest and apoptosis[32]. Furthermore, decreasing of PTEN mRNA and protein expression was found in lung cancer specimens compared to adjacent normal tissues, suggesting PTEN plays a key role in the formation of lung cancer[33]. Interestingly, our data showed that the protein expression level of PTEN was increased significantly after long-term use of tramadol, indicate PTEN might a downstream molecular of tramadol in lung cancer. In addition, upregulation of PTEN was correlated with the downregulation of phosphorylated PI3K and phosphorylated Akt, further elucidating a potential mechanism by which tramadol could be able to attenuate proliferation and metastasis of solid tumors.

There are still some limitations in our work. The sources of A549 and PC-9 were different, although they were both NSCLC cell lines. Given there might be different mechanisms in different backgrounds, our results may not be completely consistent. Therefore, the results need further in width research to confirm.

Conclusions

Taken together, our study provides new evidences that long-term treatment of tramadol inhibits lung cancer cell proliferation, migration and invasion through PTEN/PI3K/Akt signaling pathway. These results indicate that tramadol may serve as an anti-neoplastic agent involved in lung cancer pathogenesis.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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